IMPAIRMENT OF NITRERGIC RELAXATION OF THE SPHINCTER OF ODDI IN THE STATE OF TOLERANCE TO NITROGLYCERINE, IN EXPERIMENTAL HYPERCHOLESTEROLAEMIA AND LOVASTATIN-TREATMENT IN RABBITS

Ph. D. THESIS

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ABBREVIATIONS

cADPR	- cyclic adenosine diphosphate ribose
cAMP	- cyclic adenosine monophosphate
CCK8	- cholecystokinin octapeptide
cGMP	- cyclic guanosine monophosphate
CGRP	- calcitonin gene-related peptide
EDRF	- endothelium derived relaxing factor
HMG-CoA	- 3-hydroxy-3-methyl-glutaryl coenzyme A
i. v.	- intravenous
L-NAME	- N^G-nitro-L-arginine methyl ester
NANC	- nonadrenergic-noncholinergic
NG	- nitroglycerine
NO	- nitric oxide
RIA	- radioimmunoassay
SDS/PAGE	- sodium dodecyl sulfate/polyacrylamide gel electrophoresis
SO	- sphincter of Oddi
TTX	- tetrodotoxin
VIP	- vasoactive intestinal polypeptide
wt	- weight

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II. Z. Szilvassy, **R. Sari**, J. Németh, I. Nagy, S. Csati, J. Lonovics:
Improvement of nitrergic relaxation by farnesol of the sphincter of Oddi from hypercholesterolaemic rabbits.
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III. **R. Sari**, J. Nemeth , R. Porszasz, P. Horvath, I. E. Blasig, P. Ferdinandy, I. Nagy, J. Lonovics, Z. Szilvassy:
Impairment by lovastatin of neural relaxation of the rabbit sphincter of Oddi.
Eur. J. Pharmacol. 432: 91-97, 2001. ***(2.047)***

1. INTRODUCTION

Nitric oxide (NO) formed from L-arginine by a constitutive NO synthase in endothelial cells (1) was shown to play an important role in regulation of gastrointestinal blood flow (2, 3). From studies *in vitro*, NO has also been proposed as a mediator of nonadrenergic-noncholinergic (NANC) relaxation of the guinea pig stomach and ileum (4, 5), of the rat stomach, colon, duodenum and ileum (6, 7, 8, 9), of the canine ileo-cecal junction, ileum, duodenum and colon (10, 11, 12) and of human colon and internal anal sphincter (13). Pauletzki et al. (14) have shown that in the sphincter of Oddi (SO), L-arginine-NO pathways may play a major role in neural relaxation in the guinea pig. Nevertheless, regional differences in the excitatory and inhibitory innervations of the sphincter of Oddi of the latter species have also been well documented (15). Subsequently, a series of experients from our department confirmed that the L-arginine-NO pathway played a crucial role in neural relaxation of the sphincter of Oddi of the rabbit with regional dominance on the ampullary part of the sphincter (16).

Nitrergic mechanisms, however, are known to be highly susceptible to certain metabolic abnormalities e.g. hyperlipidaemia (17). It was not surprising therefore, that experimental hyperlipidaemia/atherosclerosis induced by dietary cholesterol overload significantly impaired NANC relaxation of the sphincter of Oddi of the rabbit (18). In the vasculature, functional defects have long been identified in endothelial cells in hypercholesterolaemia and atherosclerosis underlain by a decrease in the release/effect of endothelial NO (17). The reduced endothelium-dependent vasorelaxation due to hypercholesterolaemia/atherosclerosis has been proposed to result from a reduced formation and/or release of endothelium derived relaxing factor (EDRF) identified as NO. Nevertheless, the diminished nitrergic response may result from a more rapid inactivation of the NO released. Ohara et al. (19) have shown an increased superoxide anion production in hypercholesterolaemia and an improvement of EDRF-dependent relaxation by superoxide dismutase. Other mechanisms such as inactivation of NO by low-density lipoproteins was also mentioned (20). That the deficient sphincter of Oddi relaxing effect of amylnitrite, an exogenous non-enzymatic NO donor also restores with re-normalisation of serum cholesterol

level in clinical patients is in favour of changes at level of signal transduction in muscle cells. Thus, the primary aim of our work was to get an insight into mechanisms that are responsible for the impairment of nitrergic relaxation of the sphincter of Oddi produced by hypercholesterolaemia as well as to propose pharmacotherapeutic manoeuvres of possible benefit for the treatment of this particular type of biliary tract motility disorder.

2. GENERAL DESCRIPTION OF THE METHODS USED

2.1. Ethics and experimental animals

For studies on the sphincter of Oddi, we used New Zealand white rabbits. Experimental hypercholesterolaemia was induced in rabbits. The experiments performed in the present work conform to European Community guiding principles for the care and use of laboratory animals. The experimental protocol applied has been approved by the local ethical boards of Medical Universities of Szeged, Pecs and Debrecen, Hungary.

2.2. Measurement of isometric tension

Adult male New Zealand white rabbits weighing 3000-4000 g were stunned and exsanguinated. The duodenum with the sphincter of Oddi and the common bile duct were removed. Sphincter of Oddi muscle rings of approximately 4 mm length were prepared, cleaned of fat, adhering connective tissue and the underlying duodenum. Following removal of papilla Vateri, the muscle rings were mounted horizontally on two small L-shaped glass hooks one of which was connected to a force transducer (SG-O2, Experimetria, Budapest, Hungary) attached to a six channel polygraph (R61 6CH, Mikromed, Budapest, Hungary) for measurement and recording of isometric tension. The experiments were carried out in an organ bath (5 ml) containing Krebs bicarbonate buffer (mM: NaCl 118.1, KCl 4.7, $MgSO_4$ 1.0, KH_2PO_4 1.0, $CaCl_2$ 2.5, $NaHCO_3$ 25.0, glucose 11.1) which was maintained at 37°C and gassed continuously with 95% O_2 and 5% CO_2. The pH of the solution was kept constant at 7.4±0.05. The initial tension was set at 10 milliNewton (mN) and the sphincter of Oddi preparations were allowed to equilibrate for 60 min before the experiments were started during which period the sphincters developed characteristic 14-19 per min rhythmic contractions. Muscle rings with mechanical quiescence were excluded from the experiments.

Electrical field stimulation with 50 V square impulses of 0.1 ms duration was applied via two platinum wire electrodes positioned at each side of the muscle rings connected to an 'Experimetria' ST 02 (Budapest, Hungary) two channel programmable stimulator.

2.3. Determination of tissue cGMP and cAMP

Tissue cyclic guanosine monophosphate (cGMP) and cyclic adenosine monophosphate (cAMP) content was determined as described Szilvassy et al. (21). In brief, the muscle rings were instantly frozen with the use of a precooled Wollenberger clamp and pulverized in liquid nitrogen. The samples were then homogenized in 6% (v/v) trichloroacetic acid of 10 times higher quantity than sample weight in a mortar previously kept at -70°C. After thawing, the samples were processed at 4 °C. Sedimentation at 15,000 g for 10 min by means of a Janetzki K-24 centrifuge (Leipzig, Germany) was followed by extraction of supernatants with 5 ml water-saturated ether in a Wortex extractor (MTA, Kutesz, type 5191, Budapest, Hungary) over 5 min. The ether fraction was eliminated, and the extraction was then five times repeated. After the fifth procedure, the samples were evaporated under nitrogen, and assayed for cAMP and cGMP contents by use of Amersham radioimmunoassay kits (Les Ulis, France). Values are expressed as pmol/mg wet tissue weight.

2.4. Statistical analysis

The isometric tension and nerve conduction velocity data expressed as means ± standard deviation (S.D.) were evaluated with analysis of variance (ANOVA) followed by a modified t-test according to Bonferroni's method. The blood chemistry data and the levels of both tissue cyclic nucleotides were evaluated by Student's t-test for unpaired data (22).

3. Further evidence for the nitrergic nature of NANC relaxation of the rabbit sphincter of Oddi. Cross tolerance with nitroglycerin.

3.1. Concept

Several studies have revealed that tolerance to organic nitroesters is accompanied by alterations in the cGMP system in both vascular (23) and gastrointestinal tissue (24). The production of cGMP is reduced in the tolerant tissue whereas its degradation is increased (25, 26). Romanin & Kukovetz (27) have also shown that in tolerant tissue the soluble guanylate cyclase is partially desensitized. As this enzyme is believed to be a target for both exogenous and/or endogenous NO (1, 28), it is reasonable to speculate that tolerance to nitroglycerin (NG) may interfere with endogenous NO-dependent processes as well. Since this possible cross tolerance between nitroglycerin and endogenous NO had not been studied in the biliary tract, we commenced study to explore whether tolerance to NG influenced NANC relaxation in the ampullary part of the sphincter of Oddi of the rabbit in 1998 (29).

3.2. Study design

3.2.1. Isometric tension measurements

Following the equilibration period of 60 minutes the muscle rings were exposed to increasing concentrations of cholecystokinin octapeptide (CCK8) added to the organ chamber in half-log increments in a cumulative manner. A maximum contractile response to CCK8 was obtained, and the preparations were then washed repeatedly with Krebs solution until tension returned to previous baseline level. To study the effect of NG, the rings were precontracted by the EC_{50} concentration of CCK8. After a stable half maximum contractile response to CCK8 was obtained, the preparations were exposed to cumulative increases in NG concentration in half-log increments. Following washout, 6 rings were exposed to 275 μM NG for 1 h according to Silver et al (30) to induce *in vitro* tolerance to NG. The rings

were then extensively rinsed and transferred into naive tissue bath. The sphincter of Oddi preparations were then precontracted with the EC_{50} concentration of CCK8 and subsequently tested for relaxation to NG concentrations in half-log increments. These muscle rings exhibiting a significantly impaired relaxation in response to NG (see results) referred to as preparations 'tolerant' to NG. Another 6 rings were incubated with the solvent for NG in the same way. These preparations served as controls and underwent the same protocol. These preparations referred to as non tolerant ones. Following washout, the SO preparations (both tolerant and non tolerant) were subjected to electrical field stimulation. Stimulation with 50 V square impulses of 0.1 ms duration was applied via two platinum wire electrodes positioned at each side of the muscle rings connected to an 'Experimetria' ST 02 (Budapest, Hungary) two channel programmable stimulator (16). Contractile responses of the muscle rings to two consecutive trains of impulses consisting of 3 and 10 stimuli (50 V, 0.1 ms and 20 Hz) divided by a 2 minute interstimulating interval, were studied. The muscle rings were then preincubated with phentolamine, oxprenolol and atropine (all 1 μM) for 20 min. and the field stimulation protocol was repeated. These drugs were added to block adrenoreceptors and muscarinic receptors ('NANC solution'). Then N^G-nitro-L-arginine methyl ester (L-NAME), an inhibitor of NO synthase (31), at a concentration of 30 μM was added to the solution. The sphincters were subjected to the same field stimulation protocol after a 20 min. incubation with L-NAME. These procedures were followed by a successive incubation with 3 mM L-arginine (20 min.). The field stimulation protocol was repeated after L-arginine as well. Following extensive washout, the field stimulation protocol was repeated to test whether prodrug contractile responses could be reproduced. The muscle rings with altered spontaneous mechanical activity or exhibiting any significant increase or decrease in contractile responses to field stimulation (compared to control stimulations) were excluded from the study. Following the experiments, each muscle ring was subjected to histological examination by an unbiased histologist who confirmed that the SO specimens were not contaminated by adhering duodenal tissue.

3.2.2. Sampling for determination of tissue cyclic nucleotides

Sampling for cAMP and cGMP determination was done (1) after the equilibration period in the absence of any pharmacological maneuvers (control), (2) after a 20 min. incubation with 'NANC' solution, (3) when maximum contraction was obtained in response to field stimulation using 10 stimuli, (4) when maximum relaxation was obtained in response to field stimulation (10 stimuli) in 'NANC' solution. The same sampling protocol was repeated with the 'tolerant' muscle rings.

3.2.3. Drugs and chemicals

N^G-Nitro-L-arginine methyl ester, L-arginine hydrochloride, and tetrodotoxin (TTX) were obtained from Sigma Chemical Company (St Louis, USA). Phentolamine mesylate, oxprenolol hydrochloride and atropine sulphate were purchased from EGIS Chemicals (Budapest, Hungary). The compounds were dissolved in Krebs solution and added directly to the organ bath in a 50 µl volume. Nitroglycerin was purchased from Pohl-Boskamp GmbH (Hohenlockstedt, Germany).

3.3. Results

3.3.1. Effect of nitroglycerin on contractile activity stimulated by CCK8 in tolerant and non tolerant sphincter of Oddi muscle rings

The maximum increase in peak contractions produced by CCK8 was not different between the 'tolerant' and 'non tolerant' sphincters, with 29.9±5.8 and 28.3±5.2 mN (n=6), respectively. The sensitivity to CCK8 also was not different between the groups with EC_{50} (-log M) values of 8.5±0.2 and 8.3±0,1, respectively. Preparations precontracted with EC_{50} CCK8 which were exposed to a preceding incubation with 275 µM NG over 60 min ('tolerant'), exhibited a depressed relaxation response to NG relative to muscle rings preexposed to the vehicle of NG

('non tolerant') **(Fig.1/a)**. The EC_{50} concentration (-log M) values of NG were 7.4±0.2 and 5.2±0.1 mN ($p<0.01$) on 'non tolerant' and 'tolerant' muscle rings, respectively. The concentration of NG producing nearly maximum relaxation in CCK8-precontracted 'non tolerant' preparations, failed to attenuate contractions provoked by CCK8 in majority of the 'tolerant' rings **(Fig.1/b)**.

Fig. 1/a.

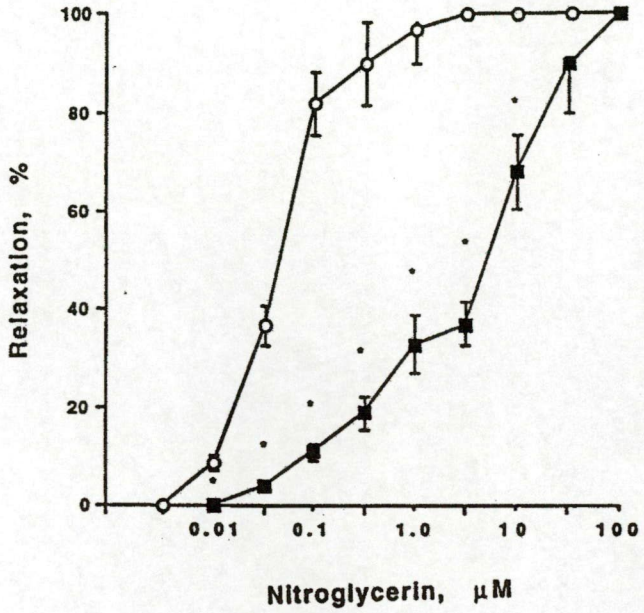

Fig. 1/b.

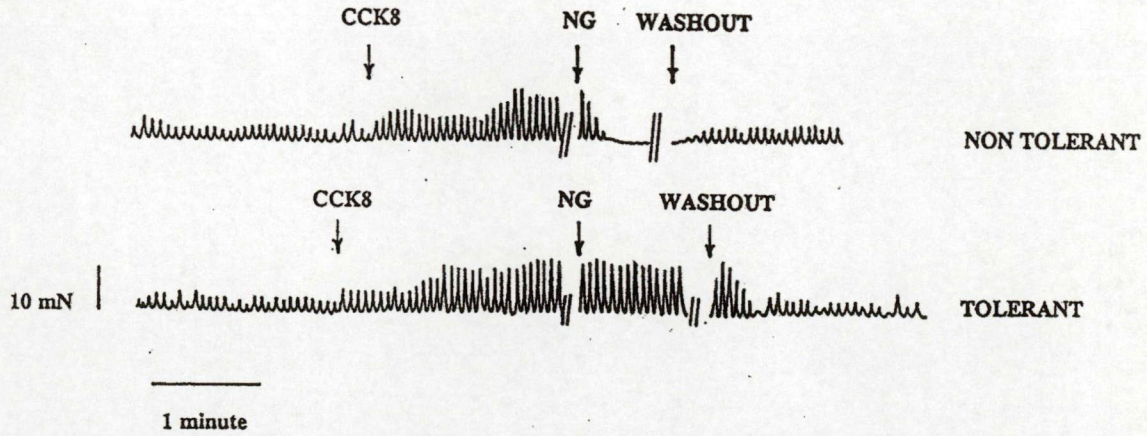

Fig. 1. Tolerance to nitroglycerin (NG) in isolated rabbit sphincter of Oddi (SO) *in vitro*. (a) Relaxation responses of SO muscle rings precontracted with EC_{50} cholecystokinin octapeptide (CCK8) to cumulative concentrations of NG. SO preparations were exposed to 275 μM NG (■= tolerant) or vehicle (○= non-tolerant) for 1h, rinsed extensively and then resuspended in naive tissue baths. Following contraction with EC_{50} CCK8, smooth muscle relaxation to added cumulative concentrations of NG was quantified. Values are the means ± standard deviation (SD) obtained from experiments with six preparations in each group. An asterisk indicates a significant difference between values obtained with tolerant and non-tolerant muscle rings at $P<0.05$. (b) Original tracing showing the effect of NG ($5x10^{-7}$ M) on contractile activity of isolated rabbit SO stimulated by EC_{50} CCK8 in tolerant (bottom) and non tolerant (top) preparations. The arrows indicate application of CCK8 or NG into the organ bath as well as the commencement of washout.

3.3.2. Effect of tolerance to nitroglycerin on NANC relaxation of the ampullary sphincter of Oddi

Repetitive field stimulation evoked twitchlike contraction followed by relaxation in the ampullary SO in both non tolerant and tolerant preparations. The magnitude of contractions and relaxations was proportional to the number of stimuli **(Fig 2/a and e)**.

Combined application of 1 μM atropine, oxprenolol and phentolamine resulted in monophasic relaxations in response to field stimulation proportional to the duration of stimulation in non tolerant sphincters **(Fig. 2/b)** but not in tolerant ones **(Fig. 2/f)**. In the presence and absence of these agents, responses elicited by electrical field stimulation were completely blocked by TTX (1 μM) and were therefore regarded as nerve responses (data not shown). Nevertheless, additional incubation with L-NAME (30 μM) reversed field stimulation-induced NANC relaxation in non tolerant muscle rings **(Fig. 2/c)** whereas the NO synthase inhibitor failed to modify stimulation-induced NANC contractions in the tolerant preparations **(Fig 2/g)**. L-arginine (3 mM) completely reversed the inhibitory effect of L-NAME on NANC relaxation in the non tolerant tissue **(Fig. 2/d)** and it was without effect on field stimulation-induced contractions in the tolerant sphincters **(Fig.2/h)**.

Fig. 2.

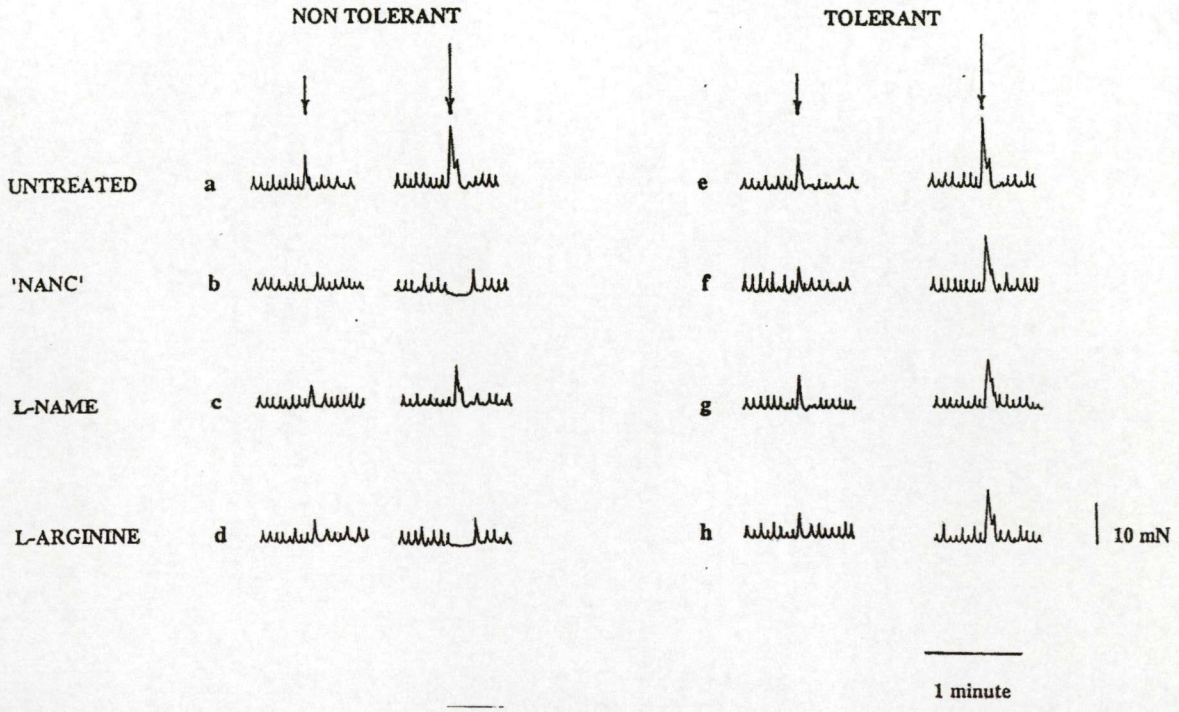

Fig. 2. Original tracings indicating the effect of electrical FS on motility of isolated rabbit sphincter of Oddi (SO). Left tracings in each plot (short arrows) represent changes in isometric tension in response to three stimuli; right tracings (long arrows) indicate changes in tension produced by 10 stimuli (50 V, 20 Hz, 0.1 ms for each). The first row (a and e) shows results obtained in SO preparations in normal Krebs solution. The second row (b and f) indicates results in NANC solution (see methods). The third row (c and g) shows the effect of N^G-nitro-L-arginine methyl ester (L-NAME) at concentration of 30 μM on NANC responses to FS. The fourth row represents findings after additional incubation with L-arginine (3 mM).

3.3.3. Effect of nitroglycerin tolerance on field stimulation-induced changes in tissue cAMP and cGMP content

Tolerance to NG was without effect on tissue content of both cyclic nucleotides measured **(Fig. 3/a and b)**. Nevertheless, NANC cAMP values in the tolerant muscle rings were lower with a border-line significance **(Fig. 3/a)**. Electrical field stimulation (20 Hz, 50 V, 10 stimuli) increased tissue cAMP and cGMP content in non tolerant preparations either untreated (Krebs solution) or exposed to NANC solution. Field stimulation failed to increase the level of either cyclic nucleotide in tolerant tissue **(Fig. 3/a and b)**.

Fig. 3/a.

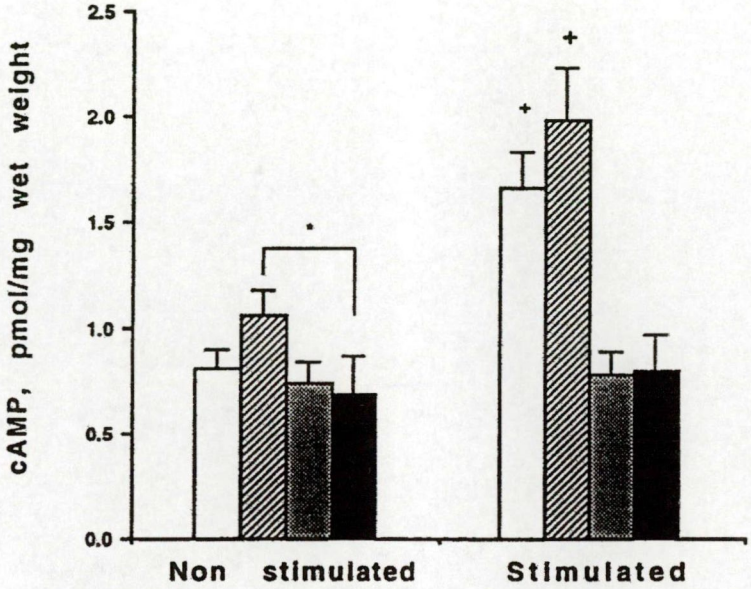

Fig. 3/b.

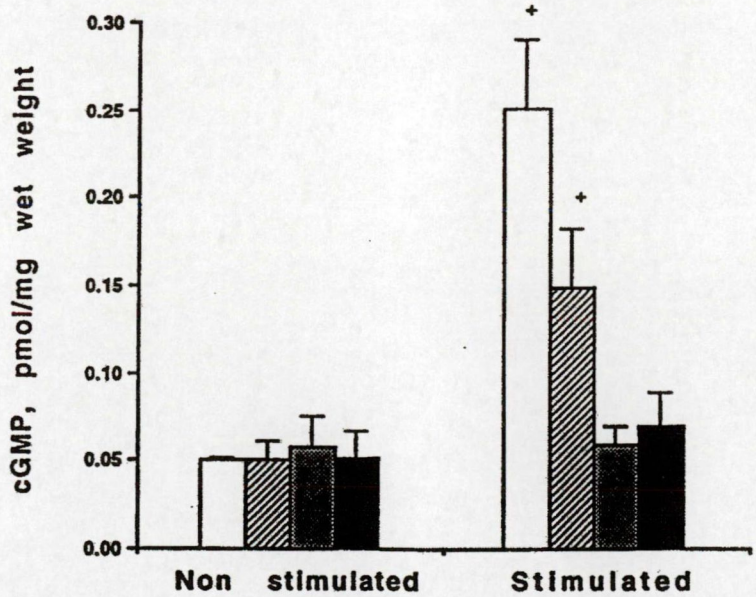

Fig. 3. Effect of electrical FS on tissue cAMP (a) and cGMP (b) content in isolated SO muscle rings of the rabbit. Open columns: muscle rings incubated in Krebs solution; hatched columns: preparations incubated with phentolamine, oxprenolol and atropine (all 1 μM, NANC solution); grey columns: muscle rings made tolerant to the relaxing effect of nitroglycerin (NG) by a preceding exposure to 275 μM NG (followed by washout) over 1h (NG-tolerant) incubated by Krebs solution; solid columns: NG-tolerant preparations incubated by NANC solution. The data are the means ± SD obtained in six preparations in each group. An asterisk represents differences within the samples in the 'non-stimulated' group at $P<0.05$. A plus signifies significant difference between corresponding values obtained from non-stimulated and stimulated preparations at $P<0.05$.

4. Impairment of neural relaxation of the rabbit sphincter of Oddi by hypercholesterolaemia. The role of farnesylation.

4.1. Background

We have shown that hypercholesterolaemia/atherosclerosis impairs relaxation function of the sphincter of Oddi in both experimental animals and clinical patients (18, 32). Moreover, the relaxant effect of exogenous non-enzymatic NO donors on the sphincter of Oddi also has been found impaired in hypercholesterolaemia (18). Since dietary hypercholesterolaemia may impair both the release and effect of NO through a deficiency in G-protein coupling at least in part due to a reduced synthesis of farnesyl or geranylgeranyl products (17), we sought whether farnesol supplementation elicited a partial recovery of nitrergic relaxation of the sphincter of Oddi preparations from hypercholesterolaemic rabbits *in vitro*.

4.2. Study design

4.2.1. Experimental groups

The study was carried out with six experimental groups (with 11 animals in each). Group 1: normal animals treated with the solvent for farnesol two times a day over three days; Group 2: normal animals treated with farnesol (30 μmol/kg body weight) two times a day over three days; Group 3: hyperlipidaemic rabbits (1.5 % dietary cholesterol load over 8 weeks) treated with the solvent for farnesol; Group 4: hyperlipidaemic rabbits treated with farnesol. Each group was divided into two subgroups: six preparations from six animals were used for isometric tension measurements, whereas five muscle rings from five rabbits were used for determination of tissue cGMP by means of radioimmunoassay.

4.2.2. Drugs and chemicals

All drugs and chemicals used in this study were purchased from Sigma (St Louis, Mo). Atropine and guanethidine were freshly dissolved in Krebs solution and added to the organ baths in 50 µl volume. Farnesol (3,7,11-trimethyl-2,2,10-dodecatrien-1-ol, mixed isomers) was diluted with 0.5 ml/kg body weight propylene glycol, therefore, propylene glycol was referred to as the solvent for farnesol.

4.2.3. Protocol for tension measurement

Biliary sphincter of Oddi muscle rings of approximately 6 mm length from adult male New Zealand white rabbits weighing from 3500-4000 g were prepared. The papilla Vateri was eliminated and the ampullary part of the muscle rings of approximately 3 mm length were mounted horizontally on two small L-shaped glass hooks of which one was connected to a force transducer attached to the polygraph. The experiments were carried out in an organ bath (5 ml) containing Krebs bicarbonate buffer as described (2.2.). Atropine (1 µM) and guanethidine (4 µM) were continuously present (NANC solution). Changes in isometric tension in response to two consecutive trains of impulses of electrical field stimulation (50 V, 0.1 ms, 20 Hz and 40 stimuli) were then studied.

4.3. Results

The cholesterol-enriched diet increased serum cholesterol to 22.6±3.8 vs pre-diet 1.4±0.3 mmol/l. In rabbits fed normal chow, serum cholesterol did not change during the same period and farnesol was without effect on serum cholesterol level.

Field stimulation-induced monophasic NANC relaxation in sphincter of Oddi muscle rings from normal rabbits treated with the solvent for farnesol. Treatment with farnesol (30 µmol/kg body weight) did not modify this response (not shown). Preparations from the solvent-treated hypercholesterolaemic rabbits responded with NANC contractions to field

Fig. 5.

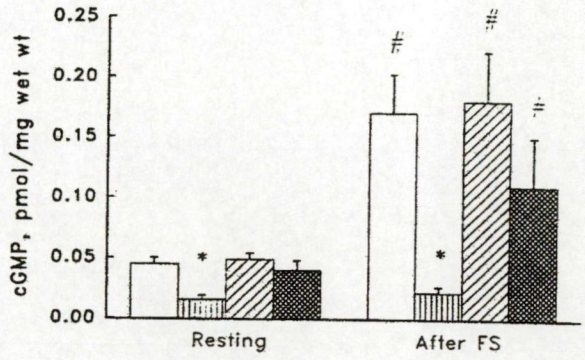

Fig. 5. Changes in cGMP content of sphincter of Oddi muscle rings in response to electrical field stimulation (0.1 ms, 50 V, 20 Hz, 40 stimuli). Open bars: rings from solvent-treated normal animals; vertical line bars: solvent-treated atherosclerotic; hatched bars: farnesol-treated normal; cross-hatched bars: atherosclerotic, farnesol-treated. Data are means ± SD obtained with five rings from five animals. *: atherosclerotic vs. normal at$P<0.05$; #: stimulated vs. resting at $P<0.05$.

5. Impairment of neural relaxation of the rabbit sphincter of Oddi by 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) reductase inhibition.

5.1. Background

As seen above, farnesol supplementation improves NANC relaxation of the sphincter of Oddi from animals with experimental hypercholesterolaemia. This is in agreement with results by Roullet et al. (33, 34) that farnesyl analogues re-normalize vascular tone deteriorated by either hypercholesterolaemia or inhibition of HMG-CoA reductase, a key enzyme in the mevalonate pathway independent of plasma cholesterol levels. Therefore, in a series of experiments we studied if pharmacological inhibition of HMG-CoA reductase by chronic administration of lovastatin decreased the NANC relaxation response in sphincters from normal animals and as to whether this could be masked by farnesol supplementation.

5.2. Study design

5.2.1. Experimental groups

Sphincter of Oddi muscle rings were prepared from groups of adult male New-Zealand white rabbits (3000-3500 g) as follows: untreated animals Group 1, and from those treated with lovastatin (5 mg/kg/day intragastrically, over 5 days) Group 2, or farnesol (1 mg/kg intravenously, twice a day for 5 days) Group 3, and from those given lovastatin + farnesol over 5 days Group 4. Each group consisted of 23 preparations from 23 animals, six of which were used for measurement of isometric tension; five rings entered radioimmunoassay studies for determination of baseline cAMP and cGMP, eight rings were used for determination of tissue NO and four rings per group served for Western blot analysis of the membrane-associated G-protein subunit $G_{s\alpha}$. Two additional groups were instituted to test the effect of the solvent for farnesol and the placebo for lovastatin (four preparations for isometric tension measurements, three for cyclic nucleotide- and four for NO determinations per group). To reduce the number

of experimental animals, field stimulation-induced changes in tissue cyclic nucleotide concentration were determined from the same preparations as those used for isometric tension measurements.

5.2.2. Methods

The measurement of isometric tension, the determination of cyclic nucleotide content in samples from isolated rabbit sphincter of Oddi and the serum cholesterol level was described (35).

5.2.3. Membrane preparations and Western blot analysis

Sphincter of Oddi muscle rings were homogenized in ice-cold 50 mM Tris/HCl (pH 7.4 at 25 °C) containing 10 μg/ml soybean trypsin inhibitor, 5 μg/ml leupeptin, 200 μg/ml bacitracin, 2 mM EDTA and 100 μM phenyl-methyl-sulfonyl fluoride to prevent proteolysis. The supernatant fraction resulting from centrifugation with 600 X g for 10 min. was re-centrifuged at 30 000 X g for 15 min. at 4 °C. The pellet was re-homogenized in fresh buffer and re-centrifuged. The final pellet was re-suspended in ice-cold assay buffer (50 mM Tris/HCl, 5 mM $MgCl_2$, pH 7.4 at 25 °C), and protein content was determined by Lowry's method using bovine serum albumin as a standard. Membrane prepararions were maintained at –80 °C for up to two weeks until utilized in assays. Sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE) and Western blotting were performed using the procedure previously described by Miyamoto et al (36). In brief, membrane suspensions were dissolved in an equal volume of sample buffer containing 62.5 mM Tris,/HCl (pH 6.8), 10 % glycerol, 2 % SDS, 5 % mercaptoethanol and 0,0025% bromophenol blue and boiled for 5 min. before application to the gel (7.5 μg protein per lane). After electrophoresis (40 mA for 100 min.), the gels were soaked for 20 min. in transfer buffer (25 mM Tris, 192 mM glycine, and 20 % methanol). Proteins were transferred from the gel to pre-soaked nitrocellulose membranes at 180 mA over 90 min. The membranes were incubated for 2 h in 0.01 M Tris/HCl, (pH

7.4)/0.9% NaCl containing 3 % bovine serum albumin. Immunodetection was carried out by incubating the membrane with specific sheep antiserum recognizing $G_{s\alpha}$ diluted 1:2000 with the above buffer overnight at room temperature. The membranes were washed 5 times over 30 min. Membranes were then incubated for 2 h at room temperature with horseradish peroxidase–conjugated goat anti-sheep IgG diluted 1:1000 with the above buffer. The antibody bound to nitrocellulose membrane was detected by the chromogenic substrate 4-chloro-1-naphtol. Immunoreactivity was also detected with the enhanced chemiluminescence Western Blot Detection System followed by exposure to Hyperfilm-enhanced chemiluminescence. Immunolabelled G proteins and the intensity of the specific bands were assayed by Soft Laser Scanning Densitometer (Biomed Instruments, USA).

5.2.4. Tissue NO determination by means of electron spin resonance

Nitric oxide content of freshly minced sphincter of Oddi tissue was measured using electron spin resonance spectroscopy after spin trapping with 55 mmol/L N-methyl-glucosamine-dithiocarbamate as described in details elsewhere. NO content was expressed as arbitrary units/mg tissue (37,38,39).

5.2.5. NANC neurotransmitter release studies

Calcitonin gene-related peptide (CGRP), concentrations were determined from 200 ml samples of organ fluid of the preparations by means of radioimmunoassay methods developed in our laboratories as described (40,41). For radioimmunoassay determination of vasoactive intestinal polypeptide (VIP) we used commercial radioimmunoassay kits (Amersham, Les Ulis, France). Sampling was done prior to (resting values) and immediately after field stimulation (at maximum contraction/relaxation).

5.2.6. Sampling for cyclic nucleotide and NO determination

The muscle rings used for isometric tension measurements were used for radioimmunoassay studies as well, to determine field stimulation-induced changes in tissue cyclic nucleotide levels. Sampling was done so that the whole preparation exhibiting contraction/relaxation in response to field stimulation was placed in liquid nitrogen in 2 s subsequent to the maximum contractile response. For control to these series of rings served those, which had not been subjected to field stimulation (resting values). The same sampling scheme was used for tissue NO measurement.

5.2.7. Drugs and chemicals

Beyond radioimmunoassay kits, all drugs and chemicals used in this study were purchased from Sigma (St. Louis, Mo) except lovastatin and its placebo (MEVACOR, Merck-Sharp & Dohme Hungaria Kft., Budapest, Hungary). Atropine and guanethidine were freshly dissolved in Krebs solution and added to the organ baths in 50 µl volume. Farnesol was diluted with 0.5 ml/kg body weight propylene glycol, therefore, propylene glycol was referred to as the solvent for farnesol.

5.2.8. *Statistical analysis*

The data representing changes in isometric tension and neuropeptide release expressed as means±standard deviation (S.D.) were evaluated by means of analysis of variance followed by a modified Student's t test for multiple comparisons according to Bonferroni's method. Changes in tissue cyclic nucleotide and NO contents were evaluated by means of Student's t test. Changes were considered statistically significant at P values smaller than 0.05.

5.3. Results

5.3.1. Isometric tension

The NANC relaxation response was converted to contraction in animals treated with lovastatin. Lovastatin-farnesol combination restored the normal NANC relaxation response. Farnesol was without effect on NANC relaxation by itself (**Fig. 6.**). The NANC relaxation was not modified by the placebo for lovastatin or the solvent for farnesol.

Fig. 6.

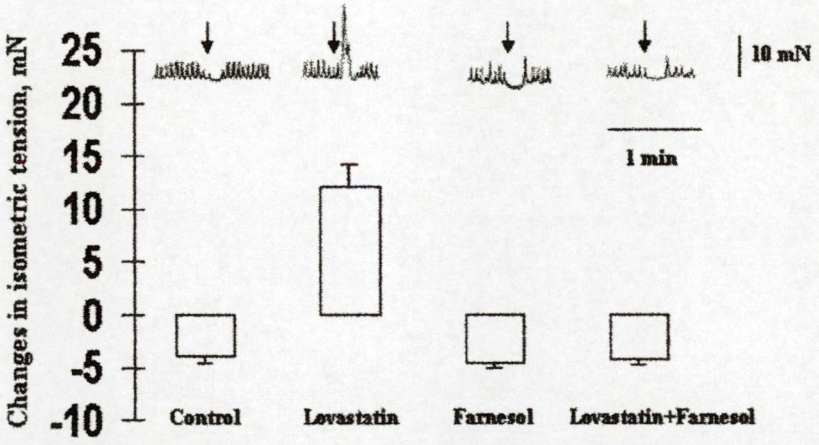

Fig. 6. Changes in isometric tension induced by electrical field stimulation (40 stimuli 50 V, 0.1 ms and 20 Hz) in sphincter of Oddi preparations *in vitro*. The data are expressed as means±S.D. obtained with 6 preparations in each group. Positive values indicate contraction, negative values denote relaxation. The original tracings in the upper part of the figure represent characteristic responses to field stimulation in each particular group. The arrows show commencement of field stimulation.

5.3.2. Tissue NO content

In tissue samples of the sphincter Oddi, neither lovastatin nor farnesol or their combination influenced either baseline or post-stimulation intensity of specific spectra of NO-L-N-methyl-glucosamine-dithiocarbamate complex assessed by electron spin resonance as compared to those from the untreated animals (**Fig. 7/a**).

5.3.3. Changes in cyclic nucleotides

Field stimulation-induced NANC relaxation was accompanied by a significant increase in both cGMP and cAMP in preparations from the untreated animals. In muscle rings obtained from the lovastatin-treated group, the increase in cGMP in response to field stimulation was much lower than that seen in sphincters from the untreated rabbits. Interestingly, field stimulation failed to increase cAMP in the lovastatin-treated group. Farnesol-lovastatin combination yielded complete restoration of the increase in both cyclic nucleotides in response to field stimulation (**Fig. 7/b and c**). Farnesol / its solvent or the placebo for lovastatin was without effect.

Fig. 7.

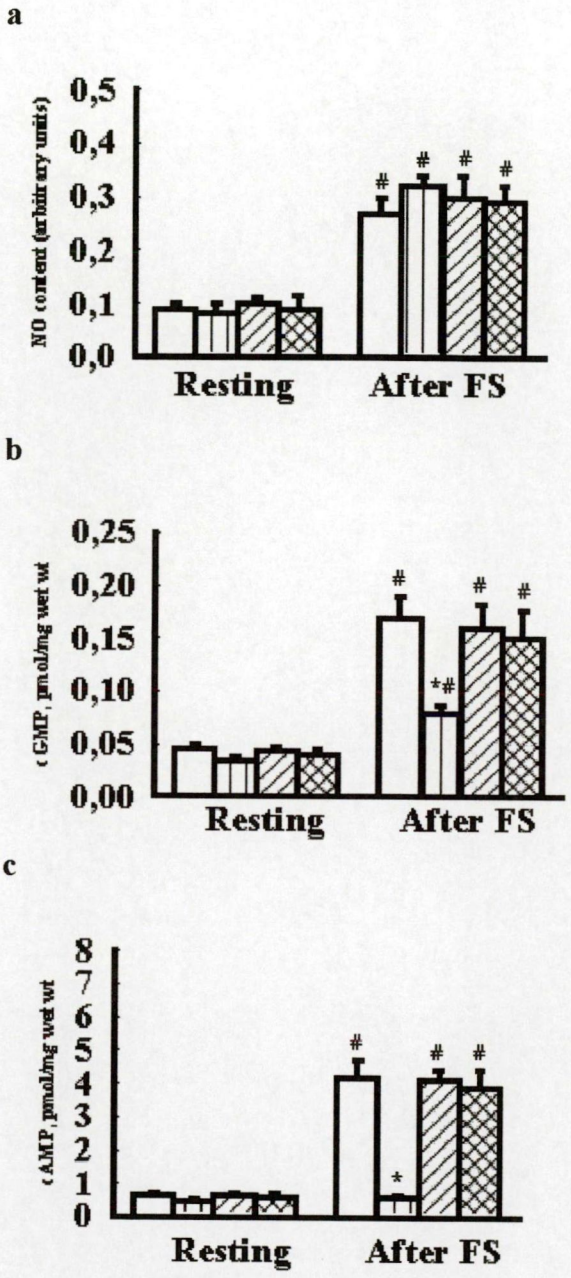

Fig. 7. Changes in tissue nitric oxide (a) cGMP (b) and cAMP (c) content of sphincter of Oddi muscle rings in response to electrical field stimulation (0.1 ms, 50 V, 20 Hz, 40 stimuli). Open bars: rings from untreated animals; vertical line bars: lovastatin-treated animals; hatched bars: farnesol-treated; cross-hatched bars: lovastatin+ farnesol-treated. Data are means±S.D. obtained with six rings from six animals. *:lovastatin vs untreated at $P<0.05$; #: stimulated vs resting at $P<0.05$.

5.3.4. NANC neurotransmitter release studies

Field stimulation induced a significant increase in CGRP and VIP concentration in organ fluid of the preparations as determined by means of radioimmunoassay. This was not modified by any of the treatments applied (**Fig. 8/a and b**).

Fig. 8.

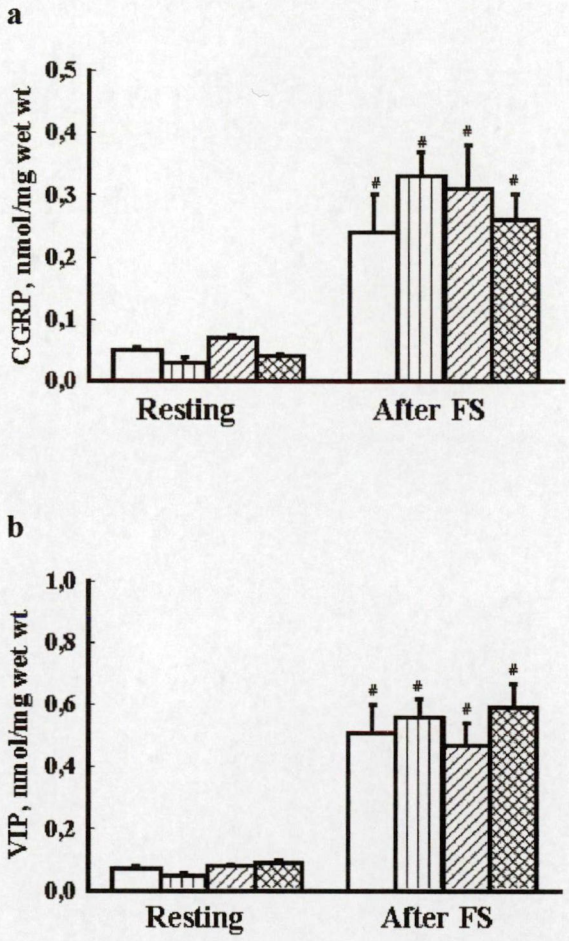

Fig. 8. Changes in calcitonin gene-related peptide (CGRP, a) and vasoactive intestinal polypeptide (VIP, b) release from isolated sphincter of Oddi muscle rings in response to electrical field stimulation (0.1 ms, 50 V, 20 Hz, 40 stimuli). Open bars: rings from untreated animals; vertical line bars: lovastatin-treated animals; hatched bars: farnesol-treated ; cross-hatched bars: lovastatin + farnesol-treated. Data are means±S.D. obtained with six rings from six animals. *:lovastatin vs untreated at $P<0.05$; #: stimulated vs resting at $P<0.05$.

5.3.5. Membrane composition of $G_{s\alpha}$ protein in the sphincter of Oddi

To estimate the effect of HMG-CoA inhibition on membrane particulation of G-proteins in the sphincter of Oddi, quantification of $G_{s\alpha}$ protein, a representative of the membrane bound G-protein complex was done by immunodetection using specific $G_{s\alpha}$ antibody. The $G_{s\alpha}$ antiserum recognized a 57 kDa band, the density of which substantially decreased in membrane preparations from sphincter from animals treated with lovastatin (**Fig. 9**). Farnesol supplementation revealed re-normalization of $G_{s\alpha}$ density in muscle from the lovastatin-treated group. Farnesol was without effect (**Fig. 9**).

Fig. 9.

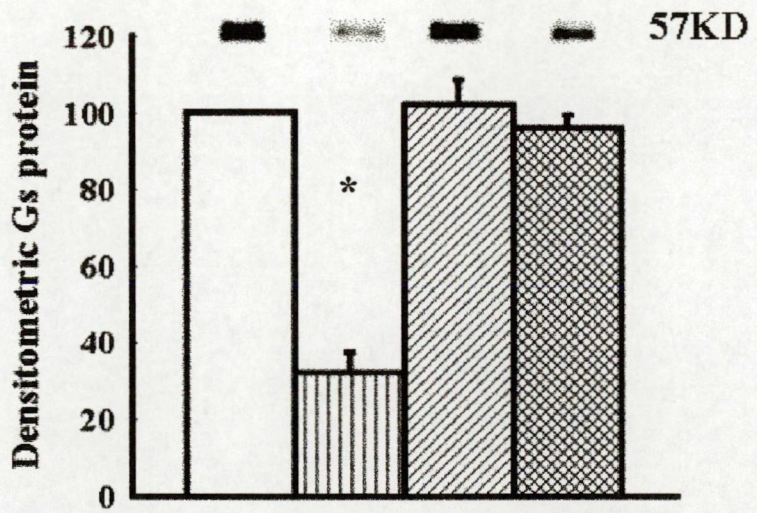

Fig.9. Western blot analysis of $G_{s\alpha}$ subunit in rabbit sphincter of Oddi membranes. 7.5 μg protein was loaded per lane. A representative gel obtained with preparations from untreated (open bars), lovastatin-treated (vertical line bars), farnesol-treated (hatched bars) and lovastatin + farnesol-treated (cross-hatched bars) animals is shown in the inset at the top of each corresponding bar. Densitometric results are expressed as a percent of density seen with preparations from untreated rabbits. Data are means±S.D. obtained with four preparations from four animals. *:treated vs untreated at $P<0.05$.

5.3.6. Serum cholesterol level

Serum total cholesterol level of 2.1±0.3 mmol/l decreased by 1.6±0.2 and 1.5±0.1 mmol/l ($p<0.01$ for both) by lovastatin and lovastatin + farnesol, respectively. Farnesol, the solvent for farnesol or the placebo for lovastatin were without effect.

6. DISCUSSION

These results confirm previous findings that the L-arginine-NO pathway is involved in the NANC relaxation of the ampullary part of the rabbit sphincter of Oddi (16). Assay of tissue cAMP and cGMP has revealed that nerve stimulation increases the content of both cyclic nucleotides irrespective of either contraction (untreated sphincters) or relaxation (NANC medium) is the physiologic response. The results also indicate that the highly reproducible NANC relaxation in the ampullary rabbit sphincter of Oddi provoked by a standardized field stimulation protocol (16) is completely lost when tolerance to the smooth muscle relaxant effect of NG develops. Moreover, in the tolerant sphincter preparations, field stimulation failed to elicit any increase in the tissue level of either cAMP or cGMP.

The nitrovasodilators are commonly used in the treatment of several diseases especially based on cardiovascular indications including ischaemic heart diseases, congestive heart failure and peripheral vascular diseases. Nevertheless, these drugs are often prescribed for non-cardiovascular patients, such as patients suffering from airway diseases, open-angle glaucoma, disturbed penile erection, achalasia, and, most frequently for the treatment of SO dyskinesis (28). The therapeutic benefit of these drugs is mainly based on their ability to relax smooth muscle. The mechanism of action is not yet fully understood, but it is strongly suggested that each nitrovasodilator must be considered as prodrug, which requires decomposition to pharmacologically active principle (42, 43) identified as NO (44). The mechanism of tolerance to organic nitroesters has not been clarified, although different hypotheses have been advanced, such as (i) tolerance caused by pharmacokinetic factors and/or (ii) the development of tolerance has been suggested to be due to oxydation of critical sulfhydryl groups in the receptor, thus converting the nitrate receptor to a low affinity state (26) and (iii) several studies have also been devoted to the question if tolerance to NG is accompanied by alterations in the formation and/or breakdown of cGMP.

The intracellular messenger cGMP is considered an important mediator of smooth muscle relaxation induced by NG. Keith et al. (24) have found an inhibition of NG-induced cGMP generation in rat aortic strips obtained from rats made tolerant to the vasodilator effect of NG by the administration of 300 mg/kg body weight NG twice daily over three days. The relaxant response to the membrane-penetrating cGMP analogue 8-bromo-cyclic GMP was,

however, not altered in the tolerant tissue, indicating that the intracellular action of cGMP, once it is formed, is not impaired. Further studies revealed that the reduced cGMP response to NG in tolerant tissue was associated with a marked decrease in the activity of the cGMP generating enzyme guanylate cyclase. The activity of the cGMP hydrolysing cGMP phosphodiesterase, however, was found slightly elevated (25). By reason of these findings, it seems possible that the development of tolerance to NG involves a cross tolerance to the effect of other exogenous and endogenous NO donors that stimulate guanylate cyclase as well. This might at least in part be responsible for the failure of field stimulation to increase cGMP in the tolerant sphincter preparations with an impairment of the NANC relaxation. However, some authors argue against the concept of desensitisation of soluble guanylate cyclase in the state of tolerance to NG (45). Interestingly, field stimulation-induced increase in tissue cAMP was also abolished in the 'tolerant' preparations. Field stimulation is a commonly used convenient method to excite neurons in various tissue preparations but evaluation of both motor and biochemical responses after such a train of electrical impulses are complex (46) as field stimulation stimulates all kinds of neurons in the preparation and only the overall response induced by stimulation of diverse population of neurons can be observed. As the sphincter of Oddi is densely innervated with NANC neurons containing vasoactive intestinal polypeptide, peptide histidine-isoleucine, neuropeptide Y, calcitonine gene-related peptide galanin, somatostatin, substance P, enkephalin, bombesin (47, 48), and NO (49), it is not easy to interpret data obtained by the field stimulation method.

The involvement of NO in NANC relaxation of the rabbit SO based on the field stimulation method has been discussed elsewhere (16). Nevertheless, besides NO, VIP is considered another putative mediator of NANC relaxation in many regions of the gastrointestinal tract that directly relaxes the smooth muscle cells with a concomitant increase in intracellular cAMP level (50). NO has been shown to enhance the release of VIP in rat colon (51) and the concept of such an interplay between the two NANC relaxants in the SO also seems to be indirectly supported by our present results. Theoretically but not exclusively NO may act to release VIP by a cGMP-dependent pathway. The possibility for cGMP-regulated neurotransmitter release comes from the evidence that cGMP stimulates the formation of cyclic ADP ribose (cADPR), a putative endogenous ligand for the ryanodyne-sensitive intracellular calcium release channels in neurons. Thus, cGMP-dependent formation

of cADPR may regulate neurotransmitter release by modulating intracellular calcium handling (52). On the other hand, VIP also can stimulate the release of NO in both neurons and muscle cells. In addition, NO produced in muscle cells can act as a 'retrograde messenger' to release VIP from nerve terminals (51). Alternatively, NO released from nerve terminals can enhance VIP release presynaptically and relax smooth muscle postsynaptically. The effects of NO at least in part are mediated by cGMP (53) wheres the release of VIP results in an increase in cAMP (50). It is therefore not surprising that both cAMP and cGMP were significantly increased by field stimulation in the non tolerant tissue. Nevertheless, the lack of effect of the same field stimulation protocol on either second messenger in the tolerant tissue with a significant impairment of NANC relaxation of the 'tolerant' muscle rings suggest a putative primary effect of cGMP in the synergism of cAMP and cGMP to produce relaxation in the rabbit sphincter of Oddi.

Our present results strongly suggest that when the formation of cGMP is inhibited by the development of cross tolerance between NG and endogenous NO (54) the production of cAMP (either VIP-dependent or VIP-independent) is also blocked in the ampullary sphincter preparations, therefore the synergistic smooth muscle relaxation attained by cAMP and cGMP is significantly attenuated. Thus, we conclude that these results together with previous findings call attention to the potential risk of attenuation of endogenous cGMP-dependent relaxation mechanisms in the state of nitrate tolerance. Considering that the most important endogenous cardioprotective mechanism i.e. ischaemic preconditioning (54, 55) was also found to be attenuated in rabbits with vascular tolerance to NG *in vivo*, we think that there is an emergency to find appropriate, clinically utilizable therapeutic regimens to reverse tolerance to NG and/or to preserve endogenous NO-dependent processes in patients maintained on prolonged nitrate therapy of whatever indication.

Our further results show that either hypercholesterolaemia or the 5-day treatment with lovastatin, a prototype of HMG-CoA inhibitors impairs neurogenic relaxation of the rabbit sphincter of Oddi, a mechanism shown to be nitrergic in nature (16). In fact, the relaxation response was converted to contraction in preparations from either hypercholesterolaemic or the lovastatin-treated animals similar to that seen after NO synthase inhibition (16). However, the present electron spin resonance studies reveal that lovastatin is without effect on NO synthesis since neither baseline nor post-stimulation tissue NO levels differed from those

observed in muscle rings from untreated animals. Interestingly, the cGMP-increase in response to field stimulation was significantly lower in preparations from the lovastatin-treated animals than that in the untreated group, with loss of the stimulation-induced cAMP-increase after lovastatin. Treatment with farnesol attained a complete restoration of both neural relaxation and the cyclic nucleotide responses deteriorated by either hypercholesterolaemia or treatment with lovastatin.

It is widely accepted that NO, through cGMP synthesis, induces a sequence of protein phosphorylation that leads to smooth muscle relaxation (1). The present results similar to that previously observed show that nitrergic relaxation of the rabbit sphincter of Oddi is accompanied by an increase in both cGMP and cAMP concentration (56). Theoretically, an increase in tissue cAMP concentration secondary to cGMP-increase might result from an inhibition of cAMP metabolism through inhibition of the enzyme type III (cGMP-inhibited cyclic nucleotide phosphodiesterase:PDE3) phosphodiesterase (57, 58). However, the simultaneous increase in tissue cGMP and cAMP concentration in response to field stimulation can better be attributed to the co-release of NO and VIP (51) the latter of which is known to cause activation of specific membrane receptors coupled to a G-protein complex for stimulation of adenylate cyclase and to increase cAMP (59, 60). Notwithstanding, cAMP may increase in response to field stimulation due to adenylate cyclase stimulation by neurotransmitters released from intrinsic or sensory nerve terminals of the sphincter of Oddi other than VIP such as CGRP (47, 61). Moreover, in addition to its smooth muscle relaxing effect, NO has been proposed to stimulate the release of VIP (and perhaps other cAMP elevating agents) from enteric nerve terminals through presynaptic mechanisms (62). VIP or CGRP once released, facilitate further NO synthesis/release through cAMP-dependent pathways, thus, an interplay between NO and these NANC peptides underlie the increase in cGMP and cAMP in the sphincter of Oddi. Our present results are in good agreement with these observations, since the field stimulation-induced NANC relaxation was accompanied by a significant increase in NO, CGRP and VIP with an ensuing increase in both cAMP and cGMP.

The major original finding of our work is that a 5-day treatment with the HMG-CoA reductase inhibitor lovastatin similar to dietary hypercholesterolaemia (18) abolished the NANC relaxation phenomenon at least under our experimental conditions. In the vasculature, functional

defects have long been indentified in endothelial cells in hypercholesterolaemia underlain by a deficiency in the release/some of the effects of NO both of which requiring the integrity of several G-protein effector systems (17). To fulfil their biological function, G-proteins must undergo a post-translation modification with farnesyl or geranylgeranyl moieties that enable them to associate with the membrane. The availability of these moieties, however, is reduced by both dietary hypercholesterolaemia (63) and as a result of HMG-CoA reductase inhibition.

Similarly, farnesyl analogues have been found to re-normalize vascular tone deteriorated by either hypercholesterolaemia or inhibition of HMG-CoA reductase, a key enzyme in the mevalonate pathway independent of plasma cholesterol levels (33, 34). Moreover, the rapid pacing-induced ischaemic preconditioning phenomenon of the isolated working rat heart, another NO-dependent process (64) has been found to be similarly deteriorated by both experimental hypercholesterolaemia and HMG-CoA reductase inhibitor treatment (65).

We therefore considered possible that a 5-day treatment with the HMG-CoA reductase inhibitor lovastatin influenced nitrergic relaxation in the gastrointestinal tract in a similar way, resulting in a deficiency of both the release and effect of NO. The present results only partially support this assumption since the release of neither NO nor that of the two relaxant peptides was attenuated by lovastatin treatment. However, possibly due to its G-protein dependence, no cAMP- increase was seen in sphincters from the lovastatin-treated group in response to field stimulation, whereas the G-protein-independent cGMP formation was only partially impaired. The relative deficiency in cGMP formation after lovastatin may reflect the possible cAMP-dependent releasing effect of VIP and CGRP on NO. This latter mechanism, however, is not evident from our results, possibly due to the semi-quantitative nature of the electron spin resonance technique used for tissue NO determination, since approximately the same NO levels were seen with or without lovastatin. Furthermore, the smooth muscle relaxing effect of cGMP has been shown to involve a G-protein-dependent activation of potassium channels that contributes to G-protein-independent relaxation pathways (66) **(Fig. 10.)**. Thus, the effect of NANC contractile mechanisms was unopposed during field stimulation in preparations from the lovastatin-treated animals in part due to a deficiency in the interplay between cAMP and cGMP elevating agents supplemented with partial loss of cGMP effects. After combined treatment with lovastatin and farnesol, the normal NANC relaxation response was re-gained with an increase in tissue cGMP content in response to field stimulation.

Fig. 10.

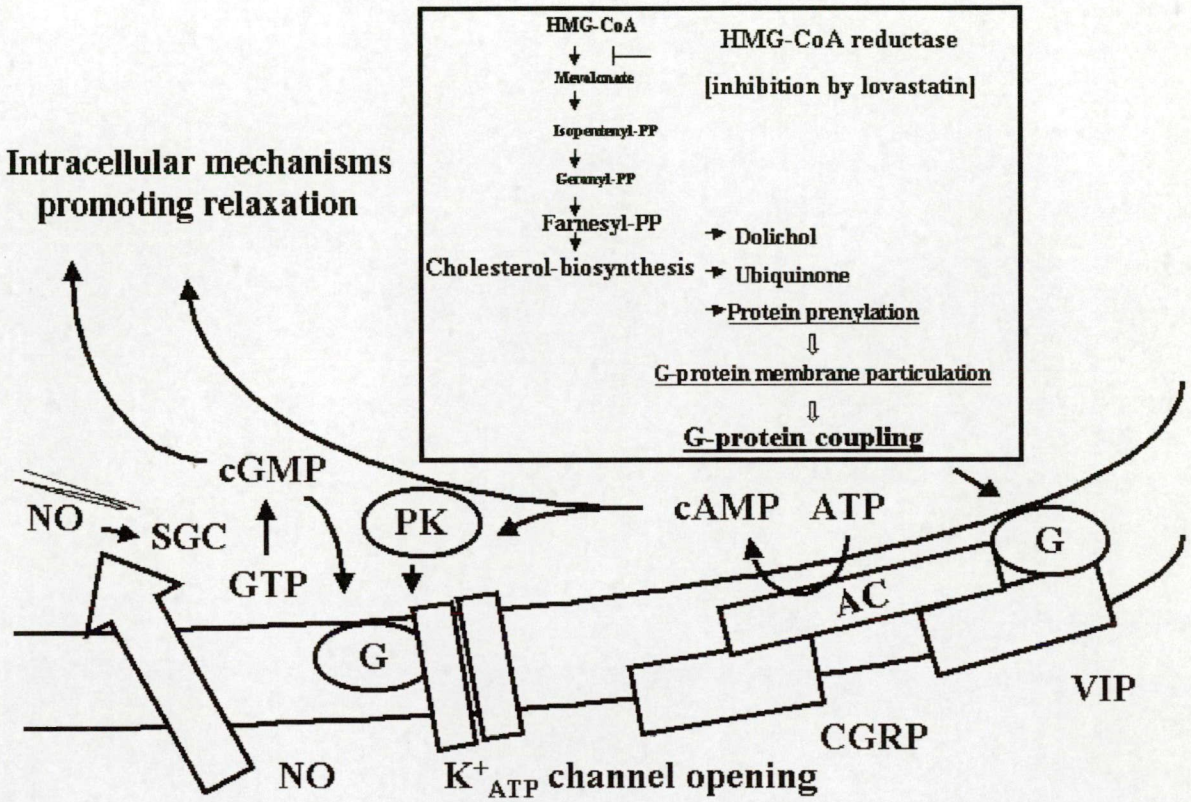

Fig.10. Schematic diagram of the link between isoprenoid biosynthesis and mechanisms underlying NANC relaxation of the sphincter of Oddi. Inhibition of HMG-CoA by lovastatin decreases formation of non-cholesterol mevalonate products such geranyl-PP and farnesyl-PP important in protein prenylation, a process that plays a key role of membrane particulation of G proteins. Thus, inhibition by lovastatin of HMG-CoA reductase results in a deficiency in G protein-dependent signal transduction pathways such as cAMP formation and activation of potassium channels.

Our data seem to support the assumption that a deficiency in the synthesis of non-cholesterol mevalonate products induced by either experimental hypercholesterolaemia or pharmacological inhibition of HMG-CoA reductase, the key enzyme of cholesterol biosynthesis impairs NANC relaxation of the sphincter of Oddi. Farnesol supplementation however, restored the relaxation response in both cases (67).

Recent studies have suggested that non-cholesterol mevalonate products are implicated in the control of vascular tone and blood pressure (34, 68). Since NANC relaxation is a pre-requisite for normal delivery of bile into the duodenum, a mechanism vulnerable to lovastatin, it is strongly suggested that non-sterol mevalonate-derived metabolites

significantly contribute to the control of extrahepatic biliary tract motility as well (67). This is supported by the fact that farnesol, the natural dephosphorylated form of farnesyl pyrophosphate that participates in protein farnesylation recaptures the normal NANC relaxation function deteriorated by either hypercholesterolaemia (67) or HMG-CoA reductase inhibition. In certain clinical cases, however, a one-month treatment with a low lovastatin dose (20 mg/kg in the evening), alleviated post-prandial right upper quadrant pain and improved the responsiveness to amylnitrite to enhance transpapillary bile flow as confirmed by results from hepatobiliary scintigraphy (32). The virtual contradiction may at least in part be explained by the difference in the degree of HMG-CoA reductase inhibition in different tissues in different species and that lovastatin may mask the effect of hypercholesterolaemia that deteriorates the relaxation function of the sphincter of Oddi by itself.

Of course, based on the present results, it is not possible to define the precise mechanism of action of farnesol to provide a beneficial influence on nitrergic relaxation of the sphincter of Oddi from either hypercholesterolaemic or with HMG-CoA reductase inhibitor treated animals.

Beyond providing further evidence that non-sterol mevalonate products participate in widespread physiological regulatory mechanisms including sphincter of Oddi function, the results call attention to the non-lipid lowering effect of HMG-CoA reductase inhibitors which should be taken into account especially with long term use of these drugs.

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9. ANNEX

Full papers published in international journals referenced by Science Citation Index